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(57) Abstract

A method of inhibiting angiogenesis and preparations for use therein are disclosed. The preparations comprise compounds thereof capable of inhibiting vascularization. The method and preparations are especially applicable to the treatment of solid tumors including skin cancers for controlling tumor neovascularization and thereby arresting tumor enlargement.

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METHOD AND COMPOSITION FOR INHIBITING ANGIOGENESIS

This application is a continuation in part of U.S. Patent No. 07/464,369 filed January 12, 1990.

GRANT REFERENCES

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FIELD OF INVENTION

This invention relates to the field of biochemistry and more particularly to methods and compositions for inhibiting angiogenesis.

BACKGROUND OF INVENTION

As a normal cell develops into a solid tumor it undergoes a series of changes. At the genetic level, oncogenes are activated and multiple tumor suppressor genes are inactivated. At the physiological level, growth is enhanced, immunity evaded, and neovascularization induced. Neovascularization appears to be a prerequisite. Experimental solid tumors are unable to grow beyond a few millimeters in thickness without a blood supply. Most natural solid tumors elaborate angiogenic factors that attract the new vessels on which they de-

pend. [For a discussion of angiogenic factors and the unsolved problem of how to inhibit tumor neovascularization, see Folkman and Klagsburn, Science, 235:442-447 (1987).] It has become increasingly evident that once a solid tumor has been established in the body every increase in tumor cell population must be preceded by an increase in new capillaries that converge upon the tumor. Consequently, there has been a continuing research effort directed toward the question of what prevents rampant capillary proliferation and what maintains the quiescent state of the capillary endothelial cells of normal tissues.

There has also been an active search for a therapeutic agent or agents which can cause capillary regression. Identification of such an agent has proven to be a very difficult problem. About the only demonstrable difference between tumor angiogenesis and other types of nonneoplastic angiogenesis is a greater intensity and persistence of the angiogenesis induced by tumors. It has become generally recognized that a therapeutic agent which can effectively inhibit tumor neovascularization should be of great value in limiting, or even completely stopping, the growth of tumors.

In one investigation of angiogenesis, Bouck, et al. carried out tests with a panel of cell hybrids derived from fusions between a chemically transformed

hamster cell line and normal human fibroblasts [Cancer Res. 46:5101-5105 (1986)]. These researchers reported that anchorage independence of the cells (which in these cells is 100% correlated with tumor forming ability) is initially suppressed and that to remain repressed they must retain human chromosome 1. These researchers further found that the suppressed hybrids were unable to elicit an angiogenic response in a rat cornea assay. In contrast, those hybrids in which anchorage independence is expressed and which have lost human chromosome 1 were found to be potently angiogenic.

Dr. Noel Bouck, Dr. Peter J. Polverini and their associates in the Departments of Microbiology-Immunology and Pathology of Northwestern University Medical School reported on further work with their hamster cell lines [Rastinejad, et al., Proceedings, 78th Ann. Meeting, Amer. Assoc. Cancer Res., Vol. 228:61, Abstract 241 (March, 1987)]. Exploration of the possibility that phenotypes of anchorage independence and angiogenesis depend on a common mediator, utilized the transforming growth factor (TGF-B). In testing for angiogenic activity, mixing experiments were performed to investigate the lack of angiogenic response to a mixture of normal baby hamster kidney (BHK) cells and a transformed cell line producing TGF-B. It was found

that the normal BHK cells or their conditioned media inhibited angiogenesis when co-introduced with transformed cells or their conditioned media into the cornea. It was concluded that secreted factors in the nontumorogenic lines can negate the angiogenic response to TGF-B.

In 1988 these same investigators (Rastinejad, et al.) reported on the finding of an inhibitor of angiogenesis under control of a cancer suppressor gene [Proceedings, 79th Meeting, Amer. Assoc. Cancer Res., 29:45, Abstract 1809 (March, 1988)]. It had been reported that in a normal baby hamster kidney (BHK) cell line, inactivation of a suppressor gene by carcinogen treatment permitted the expression of anchorage independence and tumorgenicity [Bouck and Head, In Vitro Cell and Devel. Biol. 21:463 (1985)]. Significantly, this loss of suppressor gene function coincided with loss of the ability to elaborate a factor that inhibits neovascularization in the rat cornea. Anchorage dependent revertants that were unable to suppress transformation in fusions also failed to produce the inhibitor. Using the ability of this unknown factor to inhibit bovine endothelial cell migration in a modified Boyden chamber, the factor was purified to apparent homogeneity. These results suggested that one function of the cancer suppressor gene present in the normal BHK

cells is to mediate the release of an inhibitor of the angiogenesis that is vital to progressive growth of tumors.

SUMMARY OF INVENTION

The present invention provides a method of inhibiting angiogenesis by administering a vascularization inhibitor. More particularly, the present invention is founded upon the discovery that certain peptide fragments of thrombospondin are capable of inhibiting angiogenesis.

In one embodiment peptides capable of inhibiting vascularization are provided consisting essentially of amino acids selected from sequence Id. No. 1. As expressed above, the term "consisting essentially" as used herein means that peptide includes amino acids selected from Sequence Id. No. 1, but that other amino acids may be included in the peptide that do not change the basic and novel properties of the composition.

In addition certain peptides derived from THBS-1 gene (in translation) or peptides having similar configuration have been shown to have inhibitory activity. Typical of the compositions which consist essentially of peptides suitable for use in this invention are: The Col 1 peptide (Sequence Id. No. 2),

including fragments of this peptide from both N (Sequence Id. No. 2, amino acids 1-15), and C (Sequence Id. No. 2, amino acids 11-25) terminal regions, and a short peptide ranging from amino acid 10-15 of Sequence Id. No. 2.

More specifically, this invention provides: the composition which consists essentially of peptides suitable for use in the invention are:

X - Gly - Val - Gln - Tyr - Ary - X

Wherein X are amino acid sequences which do not destroy or interfere with the inhibition of vascularization properties of the peptide such as but not limited to alanine or glycine. This amino acid sequence is shown by Sequence Id. No. 2 amino acids 10-15.

Another similar peptide pro α 1[1] shown by Sequence Id. No. 3 was also discovered to inhibit vascularization.

Another group of peptides relating to the properdin portion of TSP-1, the Mal-II Sequence Id. No. 4) and Mal-III (Sequence Id. No. 5) peptides have been found to inhibit vascularization. More specifically, portions of these peptides could provide inhibitory activity, for example, sequences such as V T X X X G V (Sequence Id. No. 8) or S P W X X X S V T X G X G V,

(Sequence Id. No. 9) wherein X represents variable amino acids that do not destroy or interfere with the inhibition of vascularization are provided by this invention. These peptides may be produced synthetically or recombinantly.

The present invention is also directed to the treatment of human patients having growing solid tumors with associated neovascularization and to the treatment of other diseases where neovascularization is a con-tributory factor to progression of the disease. A pre-ferred method of retarding the tumor growth of this invention comprises administering to the site of the patient's tumor a neovascularization inhibitor consisting of a peptide thereof capable of inhibiting vascularization. In carrying out the treatment the inhibitor should be present in the environment of the treated tumor in an amount effective for retarding enlargement of the tumor. With respect to other diseases requiring control of neovascularization, the amount of the inhibitor used should be effective for inhibiting the neovascularization at the site where it is occurring. Parenteral administration of inhibitor may also be useful in retarding or preventing growth of tumor metastases, following surgical removal of primary solid tumors, as an adjunct or replacement for conventional chemo- or radiological therapies.

DETAILED DESCRIPTION

The peptides of this invention are useful for example in inhibiting angiogenesis in vitro or in vivo. The present invention is founded upon the discovery that certain peptides posses unique and unexpected angiogenesis inhibitory activity. These peptides may be derived from TSP-2 (Bornstein et al. A second, Expressed Thrombospondin Gene (ThbS2) Exists in the Mouse Genome, J. Bio. Chem. 266:12821 (July, 1991)), TSP-1 (Hennessy et al., Complete Thrombospondin mRNA Sequence Includes Potential Regulatory Sites in the 3' Untranslated Region, J. Cell. Biol. 108:729 (Feb., 1989)), amino acid sequences encoded by genes THBS-1 and THBS-2 genes. More specifically, Col 1 (Sequence Id. No. 2), Mal-II (Sequence Id. No. 4) and Mal-III (Sequence Id. No. 5) peptides have been shown to have inhibitory activity. In the Col 1 peptide both N and C terminal regions of the peptide are active. A short peptide (comprising the overlapping region of these N and C terminal peptides ranging from amino acid 10 to 15 of Sequence Id. No. 2) may be sufficient to provide inhibitory activity.

A peptide related to Col 1, pro α 1[1] which is not derived from the TSP gene was also shown to posses angiogenesis inhibitory activity. See Sequence Id. No. 3.

Similarly, peptides Mal-II and Mal-III posses inhibitory activity. These peptides were described in Prater et al., The Properdin-like Type I Repeats of Human Thromobospondin Contain a Cell Attachment Site, J. Cell Bio. 112:1031, 1037 (March, 1991) (hereby incorporated by Reference). It is likely that not all amino acids that occur in natural TSP-1 (Sequence Id. No. 1, amino acids 131-205), are necessary for inhibitory activity. Rather a sequence such as V T X X X G V (Sequence Id. No. 10) could provide inhibitory activity. In this instance X represents amino acids. Similarly, the generic peptide S P W X X X S V T X G X G V (Sequence Id. No. 11) could provide inhibitory activity. Bornstein et al. A second, Expressed Thrombospondin Gene (ThbS2) Exists in the Mouse Genome, J. Bio. Chem. 266:12821 (July, 1991). Hennessy et al., Complete Thrombospondin mRNA Sequence Includes Potential Regulatory Sites in the 3' Untranslated Region, J. Cell. Biol. 108:729 (Feb., 1989). Similarly, for Col 1 other peptide fragments that could provide inhibitory activity include: G V or L X Y where X are amino acids which do not destroy or interfere with inhibition of vascularization.

For the above discussed peptide these compositions are typically synthetic, as this is the most efficient known method of producing a peptide in a sufficiently pure state to be highly effective and

without competing, interfering or unduly diluting components.

Proteins and active regions of proteins can be expressed in bacteria where they will not be glycosylated. They can be expressed and purified directly, as described, for example, in Sambrook et al. Molecular Cloning: a Laboratory Manual, Expression of Cloned Genes in Escherichia coli, 17:10-17:29 (1989) (hereby incorporated by reference). Alternatively, the region of interest can be expressed as a fusion protein, the fusion protein purified from the bacteria, and then the peptide of interest cleaved out and purified away from the carrier portion of the fusion protein. There are many examples of this type of strategy; one is given in Ausubel et al. Current Protocols in Molecular Biology, Vol. 2, 16.7.1-16.8.4 (1990) (hereby incorporated by Reference). There also exist ways to express the protein in yeast and in mammalian cells, but since the peptides work without glycosylation, this may not be relevant.

Known methods can be employed to provide conformational stability to these peptides so that they retain their activity upon administration to a patient. More specifically, synthetic addition to the foregoing peptides can be added which stabilizes promote or otherwise enhance their efficacy for the stated purpose.

See e.g. Pierschbachen, EPA 394,326 (hereby incorporated by reference); Braatz, EPA.453,621 (hereby incorporated by reference).

In addition to peptide fragments, nonpeptide analogs whose active or essential structural components are nonetheless based upon active structures of the natural peptides or their derivatives are also contemplated by this invention. See e.g. Debouck et al., WO 9115224 (hereby incorporated by reference); Bach et al., EPA 403,598 (hereby incorporated by reference).

By way of background, we will reiterate the experiments that lead to the original observations that human thrombospondin or fragments thereof are capable of inhibiting vascularization. We will then discuss, more recent experiments relating to the activity of specific peptides in inhibiting angiogenesis.

In developing the present invention, the BHK angiogenesis inhibitor was purified as described in Rastinejad, et al. (1989) cited above. This inhibitor was found to be a glycoprotein of about 140 kD molecular weight, which glycoprotein corresponded closely with the amino acid structure of the human thrombospondin monomer fragment from residue 294 to its carboxyl terminus. The BHK inhibitor was compared with human thrombospondin in its natural trimer form using a rat corneal assay as described in Bouck, et al. (1986), cited above. The test

materials were mixed 1:1 with Hydron (poly-2-hydroxy-ethyl-methacrylate). Small pellets containing a defined amount of the test material were implanted into the cornea of the rat eye either with or without 50 ng of the basic-fibroblast growth factor (bFGF). Positive responses were recorded when sustained growth of new blood vessels from the limbus toward the implant was observed by 7 days. Results are summarized below in Table 1.

TABLE I

Inhibition of Corneal Neovascularization By Hamster Cell gp140 Inhibitor and Thrombospondin

Test Material	Positive Corneas/Total +bFGF	-bFGF
Buffer	4/4	0/3
gp140 inhibitor		
0.025 ug	3/3	0/2
0.125 ug	0/3	0/2
0.625 ug	1/3	0/3
thrombospondin		
0.025 ug	3/3	0/2
0.125 ug	0/4	0/2
0.625 ug	0/3	0/2
2.5 ug	0/3	0/2

As shown by the foregoing data, human thrombospondin has comparable inhibitor activity to the gp140 BHK inhibitor.

Human thrombospondin (hTSP) is a glycoprotein found in the alpha granules of platelets. It is secreted from platelets upon activation by various agonists. HTSP has been extensively studied in trimer, monomer, and fragmented forms. [See, for example, Santoro and Frazier, Methods in Enzymology, 144:438-446 (1987); Gavin et al., J. Cell. Bio. 101:1434-1441 (1985); and Haverstick, et al., Biochemistry, 23:5597-5603 (1984).] The cited Majack and

Bornstein on page 59, Figure 2, diagrams the relation of the natural trimer to enzymatic degradation fragments. The human thrombospondin gene has been cloned and portions of the thrombospondin monomer has been sequenced. [See Waller and Hynes, J. Cell Biol., 103:1635-1648 (1986); Donoviel, J. Biolog. Chem., 263:18590-18593 (1988); and Hennessy, et al., J. Cell Biol., 108:729-736 (1989).]

Thrombospondin in its natural trimer form is a 420,000 kD glycoprotein. The individual monomers, which are disulfide-linked to form the trimeric structure, have a linear series of discrete functional domains that contain multiple binding sites. These domains include a heparin binding domain associated with an amino terminal end portion, a plasminogen binding domain in the central region, and a platelet binding domain associated with a carboxyl terminal end portion. Frazier, Thrombospondin: A Modular Adhesive Glycoprotein of Platlets and Nucleated Cells, J. Cell. Biol. 105:625-632.

As described by Santoro and Frazier (1987), Majack and Bornstein (1987), and Haverstick, et al. (1984), all cited above, fragments of thrombospondin can be readily prepared by enzymatic digestions. Enzymes such as chymotrypsin, thrombin, trypsin, elastase, and thermolysin cleave 25 to 35 kD segments from the amino end of the monomers. The remaining 140 kD fragments

remain united in trimer form. When chymotrypsin or thermolysin is employed for the enzydigestion, the protein is also reduced to 120 kD fragments, which not only have their amino ends trimmed as described but also have an 18-25 kD segment trimmed from their carboxyl ends. The 25-35 kD fragment contains a heparin binding site while the 18-25 kD fragment contains a platelet/cell binding site. A mixture of both the 120 kD and the 140 kD fragments can be produced in the same procedure.

The 140 kD and 120 kD fragments are usually obtained in trimer form, and can be resolved into monomer form by incubation with dithiothreitol. When the trimer of the 140 kD fragment or the trimer of the 120 kD fragment is enzymatically treated with chymotrypsin in the presence of EDTA (ethylenediaminetetraacetic acid), two further fragments are obtained, a 70 kD fragment extending from the amino end of the 120 kD fragment and a 50 kD fragment also extending from the amino end of the 120 kD fragment. The 50 kD/70 kD fragments contain the region(s) that functions as an angio-genesis inhibitor, and are usable for the purposes of the present invention. This being true, it is to be ex-pected that further fragmentation of the 70 kD fragments should be possible.

Methods exist for confirming whether or not the inhibitor activity against neovascularization is retained, thus making it easy to confirm usefulness for the purposes of this invention. Available methods include the rat corneal assay. Selected fragments can be produced by genetic engineering methods as recombinant fragments, since the gene and cDNA for hTSP has been isolated and cloned (citations given above). Other variations are also expected to be feasible. These include deglycosyl-ated forms of HTSP trimer, monomer, or fragments, which can be prepared by known procedures for deglycosylating glycoproteins. [See Edge, et al., Analyt. Biochem., 118:131-137 (1981) for a deglycosylation procedure using trifluoromethanesulfonic acid.] Alternatively, the HTSP gene for the monomer or gene segments for fragments thereof can be expressed in cells which do not result in glycosylation of the monomer. Although it may be preferred to use glycosylated forms of the hTSP inhibitors of this invention, this is not believed to be essential. Furthermore, for production of inhibitors by biochemical or by genetic engineering procedures, deglycosylated forms may be less expensive than glycosylated forms.

Since thrombospondin is a multiple domain glycoprotein having other bodily functions, it is pre-

ferred to administer the inhibitors of this invention by extravascular procedures. For example, hTSP is known to cause or augment platelet aggregation, and therefore in its natural form it would be inadvisable to administer hTSP by a parenteral route. However, by trimming hTSP fragments as described above, or by using a selected sequence of the hTSP gene to produce trimmed forms, inhibitors can be produced which contain primarily the active region which inhibits angiogenesis. It may therefore be safe to administer such modified fragment forms by parenteral routes.

Effective extravascular routes are available. For internal tumors, the inhibitor can be directly implanted by known techniques. For example, it can be combined with slow release polymers such as poly-2-hydroxyethylmethacrylate or methylenevinylacetate copolymer. When combined with such retardants, the inhibitor can be prepared in the form of pellets of known inhibitor content, and selected quantities of the pellets can be directly implanted in the tumor. For skin tumors, which are also classified as solid tumors, the inhibitor can be combined with a topical ointment and applied directly to the surface of the tumor. Procedures for preparing such administration vehicles whether for pellet implantation or for direct surface

application are further described in the following examples. Other procedures which may be useful include the preparation of the inhibitor in aerosol form for application to tumors at the respiratory tract or lung, using standard devices employed by respiratory therapists to deliver aerosols.

In general, it is believed that the inhibitors of this invention will be of greatest value for arresting the growth of rapidly growing tumors like melanomas. However, all solid tumors are dependent for growth on the generation of new capillary vessels, and the method of this invention is believed to be generally applicable to internal solid tumors and to all forms of cancer growing in the skin. When a solid tumor is surgically removed, an implant may be placed at the site of the removed tumor, thereby inhibiting the angiogenesis of any reforming tumor at the same site.

The required dose for lessening enlargement of a tumor will vary with the size and location of the tumor. Amounts may range from 1 microgram (ug) to 1 milligram (mg). It is believed that preferred amounts will usually range from 100 ug to 800 ug quantities per dose. In general, an amount will be applied to the site of the tumor sufficient to retard growth of the tumor. The amount required for this purpose can be monitored by standard procedures. Where the tumor is still growing

despite the application of the inhibitor, additional quantities will be administered. Preferably, a sufficient dose is utilized to substantially stop the increase of tumor size, or in some cases to decrease the size of the tumor. Such a result can be observed by a number of methods, depending on the location and type of tumor involved. These methods include: visual observation of surface tumors, palpitation, radiological measurement (viz., X-rays for lung tumors, mammograms for breast tumors, etc.), use of ultrasound with computer assisted tomographic scanners (CAT scans), magnetic resonance imaging, radionucleotide scanning, and other standard clinical techniques used to monitor particular tumor types.

In addition to solid tumors, the inhibitors of this invention may be used as therapeutic agents for other diseases involving angiogenic dysfunction. These diseases include diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, Kaposi's Sarcoma, endometriosis, and unwanted scar formation in wound healing and unwanted granulation tissue formation after injury such as burns and prior to transplants of

cultured cells or tissues over such injuries. The amount to be used should be sufficient to partially or completely prevent the angio-genesis at the site where it is occurring. See Moses et al., Inhibitors of Angiogenesis, Bio/Tech. 9:630-634 (1991).

Similarly, syndromes such as Li-Fraumeni syndrome, wherein an increased susceptibility to tumor formation is inherited or arises as a result of a mutation in a gamete, may be treated with the present inhibitor. Both dominantly and recessively inherited syndrome are known in which afflicted individuals are at a vastly increased risk of solid tumor development. An individual with such susceptibility could by given anti-angiogenic therapy at an early age.

The preparation of inhibitors useful in the method of the present invention and their modes of administration are further illustrated by the following examples.

EXAMPLE 1

Preparation of Pure Human Thrombospondin (HTSP) From Whole Blood

Whole human blood, preferably freshly collected in ACD (8 g/L citric acid, 22 g/L trisodium citrate, 24.5 g/L glucose, pH, 4.5), 9 parts blood to 1 part ACD, is centrifuged at 180 x g for 15 minutes at room temperature. The platelet rich plasmia (PRP) is transferred to a new tube and 1/5 volume of ACD is

added. The platelets are pelleted by centrifugation at 1100 x g for 10 minutes at room temperature, and then resuspended in buffer containing 0.15 M NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na2HPO4, 24 mM NaH_2PO_4, 5 mM glucose, pH 6.5. The platelets are washed twice in the above buffer, and then resuspended in 8 ml of TCS buffer (0.15 M NaCl, 0.02 M Tris, pH 7.6, 1 mM $CaCl_2$) containing 5 mM glucose per unit of platelets. The washed platelets are activated by addition of 0.5 U/mL of human thrombin (Sigma) and incubated at 37°C for 1-2 minutes, until large aggregates are formed. The reaction is stopped by addition of 4 U/mL hirudin and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The material is centrifuged at 1000 x g. Fibronectin is removed from the supernatent by chromatography on gelatin-sepharose (Pharmacia) equilibrated with TCS buffer at 4°C. The flow through material is collected and loaded onto a heparin-Sepharose column (Pharmacia) equilibrated with TCS buffer, and washed with 0.25 M NaCl and the thrombospondin eluted by increasing the NaCl concentration to 0.6 M. To remove low molecular weight contaminants the eluent is loaded onto a Bio-Gel A 0.5 m column (Bio Rad Laboratories) equilibrated with TCS buffer. The thrombospondin elutes in the void volume and is stored at -70°C in TCS buffer containing

20% w/v sucrose. [See Santoro and Frazier, Methods in Enzymology, 144:438-446 (1987) for analogous method.]

EXAMPLE 2

Reduction of Pure Human Thrombospondin (HTSP) to Its Monomeric Form

Pure thrombospondin trimer is incubated in 25 mM dithiothreitol (DTT) (or higher DTT concentration if found to be necessary) at 37°C for 1 hour. The DTT is then removed from the thrombospondin preparation by dialysis against two changes of a 1000x excess of TCS buffer at 4°C. A portion of the preparation is checked for complete reduction of the 450kD trimeric form to the 180kD monomeric form on a 6% non-reducing polyacrylamide gel.

EXAMPLE 3

Derivation of the 140kD and 120kD Fragments of Human Thrombospondin

To produce a 140kD fragment of thrombospondin from the 180kD, the calcium-replete monomer is treated with 4 U/mL thrombin for 1 hour at 37°C. Alternatively, the calcium-replete thrombospondin monomer can be treated with L-1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin (TPCK-treated trypsin) at an enzyme to substrate ratio of 1:20 for 5 minutes at 22°C. The reactions are stopped using 1mM diiodopropyl fluorophosphate (DFP) for thrombin. The fragments can

be further purified by gel chromatography over a heparin-Sepharose column equilibrated with TCS buffer at 4°C. The 140kD fragments, lacking the N-terminal heparin binding domain, will elute in the flow through, whereas undigested whole thrombospondin will be retained. Alternatively ion exchange chromatography can be used to separate the two species.

The 120kD fragment lacking both the N-terminal 30kD and the C-terminal 25kD domains is produced by treatment of thrombospondin with 4 U/mL thrombin for 120 minutes at 22°C. The reaction is stopped as above. [See Lawler, et al., J. Cell. Biol. 260:3762 (1987) for analogous method.] For each of the above reactions, the desired fragments can be further purified by ultrafiltration through a YM membrane (Amicon) or by gel filtration.

EXAMPLE 4

Derivation of Thrombospondin 70kD Subunits

To produce 70-kD monomeric fragments of thrombospondin, calcium replete thrombospondin is dialized into TBS (0.02 M Tris, pH 7.6, 0.15 M NaCl) containing either 5mM EDTA or 10 mM Mg^{++} [Dixit, et al., J. Biol. Chem. 261:1962 (1986)] followed by digestion of this calcium-depleted thrombospondin with 0.5% chymotypsin (wt/wt) (Sigma) for 15 minutes at

25°C. The reaction may be terminated as described in Example 3 or by other suitable reagent. The digest is applied to a Sephadex G-100 column equilibrated with TBS and the 70 kD trimer eluted from the column [Galvin, et al., J. Cell Biol. 104:1413 (1987)].

EXAMPLE 5

Reduction of the Trimeric Fragments of Thrombospondin to the Monomeric 140 kD, and the 120 kD and 70 kD Fragments

The 140 kD, 120 kD, and 70 kD fragments if in trimer forms can be reduced to their monomeric forms using the procedure of Example 2.

EXAMPLE 6

Deglycosylation of Intact Thrombospondin and Its Trimer or Monomer Fragments

N-linked oligosaccharides can be removed by the following protocol. A 20 ug sample (2 ug/uL) of whole thrombospondin or one of its fragments, isolated as described above in 0.5% SDS, is incubated for 1 hour at 37°C. Following this incubation, 10.8 ul of 0.55 M sodium phosphate, pH 8.6, is added along with 3 uL of 100 mM 1,10-phenanthroline hydrate in methanol, and 5 uL of 7.5% NP-40. N-glycanase (250 U/mL, Genzyme) is added to a final concentration of 2.5 u/mL. The sample is in-

cubated for 2 hours at 37°C. Following this incubation, the sample is checked for loss of carbohydrate moiety by analysis on 6% SDS-polyacrylamide gels [Laemmeli, Nature, 227:680 (1970)]. The unglycoslyated sample is applied to a Superose gel filtration column (Pharmacia) equilibrated with PBS at 4°C and proteins are eluted in the same buffer. The major protein peak at the molecular weight corresponding to approximately 6% less than that size is expected from the glycosylated peptide taken, and dialyzed against TCS using a centricon-30 microconcentrator. An alternate method that can be used in conjunction with the above method to remove o-linked carbohydrates is described in Edge, et al., Analyt. Biochem., 118:131-137 (1981).

EXAMPLE 7

Procedure for Preparing Injectable or Implantable Slow Release Pellets

Microgram quantities of hTSP (monomer or trimer) prepared as described in Examples 1 and 2, or an active TSP fragment prepared as described in Examples 3 to 6 are incorporated into one of several slow-release noninflammatory polymers. The two most often used are poly-2-hydroxylethyl-methacrylate (Hydron^{R} Lot No. 110, Interferon Sciences, Inc., New Brunswick, N.J.) and

ethylene-vinyl acetate copolymer (EVA, Aldrich Chemical, Milwaukee, WI). Both materials work with equal effectiveness. [See Langer and Folkman, Nature, 263:797-800 (1976), for descriptions of preparations and use of these retardants.]

For example, sterile casting solutions of Hydron^R are prepared by dissolving the Hydron powder in absolute ethanol (12% w/v) at 37°C with continuous stirring for 24 hrs. An equal volume of Hydron and the active agent (e.g., hTSP) (50%) are combined and 10 ul of solution are pipetted onto the surface of a sterile 3.2 mm diameter, 1.2 cm long Teflon^R (DuPont Corp.) rods glued to the surface of a petri dish. After drying for 1-2 hrs the approximately 2 mm diameter disks can then be stored at 4°C or can be implanted immediately.

Alternatively EVA pellets are prepared by dissolving them, 40% by weight in methylene chloride at 37°C. The active agent (e.g., hTSP) is then added to the EVA solution and small (10 ul) quantities are pipetted into glass molds and air dried under vacuum. Dried pellets are washed extensively, 10-15 changes, in methanol to remove any free methylene chloride. The pellets are then ready for administration.

Alternatively, the peptide may be administered systematically. The peptides, in contrast to the whole

molecule, will cross from the blood stream into the interstitial space, especially in the area of a growing tumor and be effective.

EXAMPLE 8

Administration of Slow Release Pellets

Pellets prepared as described in Example 7 can be implanted into solid tumors with the use of a wide bore (16 gauge) trochar for the precise positioning of the pellets. Under anesthesia, pellets containing 1 ug to 1 mg quantities of TSP monomer, or trimer, or active fragments are loaded into the barrel of the trochar. Several pellets can be positioned at a single location or at multiple sites within the tumor. To study the potency of the inhibitory response, angiographic studies are performed to assess whether there has been any retardation or regression of tumor vessels. Once the tumor has stopped growing or has undergone a marked reduction in size, it can be removed. Alternatively implantation can be repeated, particularly with large tumors where prolonged exposure to the inhibitor is necessary to reduce the tumor size.

EXAMPLE 9

Treatment of Skin Cancers

The inhibitors described herein can also be formulated into ointments or suspensions in combination

with purified collagen in order to produce semisolid or suspension vehicles. Conventional oleaginous formulations containing the inhibitor can be used as salves. Such formulations will release the inhibitor on a sustained basis at the skin cancer site.

Purified human skin collagen obtained from commercially available sources can be used in the ointments. The inhibitor can be incorporated into the collagen solution where under alkaline conditions the collagen inhibitor solution will gel. Thin films can be prepared by dispensing the liquid collagen inhibitor atop glass plates. The thin sheets can then be placed atop a skin surface growing cancer and covered by a semipermeable membrane to allow for air exchange. Alternatively, the inhibitor can be incorporated in one of several petroleum jelly-based materials along with dimethyl sulfoxide to increase absorption of the inhibitor into the skin tumor, and applied as a salve where it could be applied several times a day to the surface cancer. It may be necessary to scarify the surface of the tumor to enhance penetration of the inhibitor into the neoplasm.

The types of surface cancers where this might be useful include but are not limited to basal cell carcinoma, squamous cell carcinoma and melanoma.

The frequency of topical application can be judged empirically by periodically checking for reduction in tumor size.

EXAMPLE 10

Test Procedures to Confirm the Inhibitor Effect of TSP Fragments

Human thrombospondin fragments prepared from human platelets or prepared from segments of the human thrombospondin gene can be tested to confirm inhibitory activity against angiogenesis by a number of established procedures. All of the following procedures are believed to be usable for this purpose. To confirm the inhibitory effect determination, two or more of the following assays can be used.

A. Assay of TSP Fragments for Inhibitory Activity in the Rat Cornea

The surgical procedure used to form a corneal pocket is essentially identical to that first described by Gimbrone, et al., J. Natl. Cancer Inst. 52:413-427 (1974), for rabbit cornea. Imbred F344 rats are available for routine use, but any rat strain is suitable. Male or female rats weighing 150-200 gm are anesthetized with sodium pentobarbital (29 mg/kg body wt). The eyes

are gently proptosed and secured in place by clamping the upper eyelid with a nontraumatic hemostat. Using a No. 11 Bar Parker blade a 1.5 mm incision is made approximately 1 mm from the center of the cornea into the stroma but not through it. Depending on experience this procedure can be done with or without the use of a dissecting scope. A curved iris spatula (No. 10093-13, Fine Science Tools, Inc., Belmont, CA) approximately 1.5 mm in width and 5 mm in length is then inserted under the lip of the incision and gently blunt-dissected through the stroma toward the outer canthus of the eye. Slight finger pressure against the globe of the eye helps steady it during dissection. The spatula is premarked so that the shaft does not penetrate laterally into the stroma more than 2.5 mm. Once the corneal pocket is made the spatula is removed and the distance between the limbus and base of the pocket is measured to make sure it is no closer than 1 mm. The pocket base is typically between 1-1.5 mm from the limbus. (Extending the pocket depth any closer than this often results in a false positive response. Also if the depth of incision is too close to the inner surface of the cornea nonspecific inflammation invariably occurs.)

The first 24-48 hours after implantation are critical. If nonspecific inflammation is to occur it

will manifest during this time. In such cases corneal clouding and the presence of a yellowish exudate signals inflammation. As long as asepsis is maintained and trauma during surgery is minimized, nonspecific inflammation will rarely be a problem. Even in the most carefully executed procedure some transient corneal edema will occur. However, this usually resolves within 24 hrs. Just before implanting Hydron or EVA pellets containing the pure TSP fragments with or without an added inducer at angiogenesis being tested, the pellets are rehydrated with a drop of sterile lactated ringers solution. Pellets are then positioned down to the base of the pocket which then seals spontaneously. No more than half of the pocket should be occupied with implant material. More than this the resultant transient edema will cause spontaneous expulsion of the implant. Corneas are examined daily or on alternate days with the aid of a stereomicroscope to monitor responses.

Scoring of Vascular Responses: The corneal bioassay is sometimes regarded as a qualitative assay, but a numerical quantitative measure has been devised for use with this model system. Responses are usually scored on the day animals are killed, 5-7 days after implantation. Positive responses are recorded when sustained ingrowth of capillary loops or sprouts is detected. Negative scores are assigned to responses where either no growth

is detected or when an occasional sprout or hairpin loop is detected without evidence of sustained growth is observed. Occasionally (10% of the time) responses are encountered that are neither unequivocally positive nor negative with samples which are normally positive or inhibitory. In these instances, the responses can be graded. Several factors account for this, i.e., slight differences in the position of implants within the cornea, variations in the quantity and quantity of material being tested. (This technique inherently involves a degree of variability, but this can be kept at a minimum).

Preparation of colloidal carbon perfused whole mounts: Permanent records of vascular response are made following perfusion with india ink. Commercially available waterproof india ink is usable. Perfusion is accomplished with a simple pressure vessel capable of maintaining a pressure of 120 mm/Hg. Perfusion via the abdominal aorta is carried out, introducing 100-200 ml warm (37°C) lactated ringers solution per 150 gm rat. Once the animal's snout has completely blanched, approximately 20-25 ml of ink is injected until the head and thoracic organs have completely blackened. Eyes are then carefully enucleated and placed in half-strength Karnovsky's fixative or neutral buffered formalin for 24 hours. The next day cor-

neas are dissected free from the surrounding globe and underlying iris, are bisected and loosely mounted between two glass slides where they are gently flattened. Corneas are photographed with a dissecting microscope equipped with a camera. If desired, the response can be quantitated. For example, vessel length can be measured directly using 4x5 transilluminated photographic negatives at a magnification of 10x. Three radially oriented measurements are taken using a vernier caliper; two of these measurements include vessels present at the periphery of the radius and the third includes the largest vessels along the center of the radius. The three measurements are averaged to provide a single length of measure for each response. Differences between groups are compared using a students t-test.

B. Assays for Angiogenic Activity In Vitro.

Endothelial Cell Chemotaxis: Endothelial cell migration can be assayed using a 48-well modified Boyden chamber (Nucleopore Corp) equipped with Nucleopore membranes (5 u pore size) that have been soaked overnight in 3% acetic acid, incubated for 2 hours in 0.1 mg/ml gelatin or matrigal, rinsed in sterile water, dried under sterile air, and stored for up to 1 month. BCE cells usually not older than passage 10 are preferred. (Older cultures may show greater variability in

responsiveness to chemotactic stimuli. Also, differences in the re-sponsiveness of endothelial cells to chemotactic stimuli vary from isolate to isolate so comparisons should be made within each experiment.)

Chambers are set up using 5×10^5 cells suspended in 1.5 ml of DME media supplemented with 0.1% FBS. The bottom wells are filled with 25 ul of the cell suspension and covered with the gelatin-coated membrane. The chamber is assembled and then inverted and incubated for 2 hrs to permit adherence of BCE to the membrane surface. This modification of the standard assay is important since the inhibitory activity may interfere with adhesion of BCE to the membrane surface. The chamber is then turned upright, 50 ul of the TSP fragment (dose response curve show that 5nM concentration gives an excellent response for all active fragments) along with 10 to 50 ng of FGF is dispensed into the top wells, and the chamber is incubated for an additional 2-4 hrs. The membrane bound cells are carefully washed with buffered saline, stained with Diff-Quick stain (American Scientific Products), and membranes are mounted with Permount^R (Fisher Scientific) or taped to the slide with the surface to which the cells have migrated up. The number of cells which have migrated per 10 high powered (x100) fields are counted in four independent wells. By focusing through the membrane it is possible

to distinguish the surface of the membrane to which cells have migrated.

Peptides are assayed similarly over a wide range of concentrations and the dose (micromolar concentration) giving 50% of maximum inhibition determined. Peptides requiring more than 100 uM concentration to inhibit migration are considered ineffective. Such a conclusion is verified by a cornea assay. Peptides that are 50% effective at doses = or less than 5 uM are considered effective inhibitors, a conclusion also verified in the cornea. Peptides are tested in vivo in the cornea at a concentration of 100 to 500 uM in the pellet.

Migration assay is modified for collagen peptides in that BCE cells are incubated for 24-48 hours prior to use in a regular migration assay with the peptides being tested at 20 micromolar. This allows time for delayed effects to take place. Pre-treated cells are used in a migration assay with the peptides again at 20 uM.

Table II

INHIBITORY ACTIVITY OF THROMBOSPONDIN PEPTIDES

Peptide	-	1	2	3	4	5	6
Inhibition of migration[a]	-	c	>100	0.6	5	>400	300
Inhibition of angiogenesis[b]	9/9	0/4	3/4	0/4	0/8	3/3	nd

a micromolar ED_{50}

b positive corneas/total injected with peptide and bFGF at 50 ng per pellet.

c see Table III

Peptides 1 corresponds to the col 1 peptide. Peptide 2 is MAL I, peptide 3 is MAL II, Peptide 4 is MAL III, peptide 5 is from the calcium binding region of TSP-1, and peptide 6 is from the cell attachment region of TSP-1.

Table III

IN VITRO MIGRATON ASSAYS OF COL PEPTIDES

peptide (20 uM)	% maximum migration of endothelial cells	
	peptide alone	peptide + bFGF (10 ng/ml)
col 1	<1	<1
col N	1	<1
Col C	<1	<1
pro-al(1)*	<1	<1
none	1	100

Bovine capillary endothelial cells were pre-treated for 36 hours with peptide to be tested at 20 uM, then harvested and tested in a usual migration assay in the presence of peptide with or without bFGF to induce migration.

* For this peptide only the assay was varied by soaking the membrane across which the cells migrate in Matrigel instead instead of gelatin.

Table IV

IN VIVO CORNEAL ASSAYS OF COL PEPTIDES

peptide	Positive corneas/total injected	
	peptide alone	peptide + bFGF
col 1	0/3	0/4
col N	0/2	0/3
col C	0/3	0/4
none	---	19/19

Usual rat cornea assay with peptide at 100 to 200 uM and b FGF at 50 ng/pellet.

C. Chicken Chorioallantoic Membrane (CAM) Assay:

Fertile eggs are incubated in a stationary position for 3 days at 37°C and 70-80% relative humidity. During this time, the embryo rises to the upper surface of the egg contents. At the beginning of the 4th day, the eggs are cracked without inversion and carefully deposited into sterile plastic petri dishes such that the embryo remains on the upper surface. The shell-free eggs are incubated for an additional 72 hours at 37°C, under an atmosphere containing 2.5-3.5% CO_2, after which the growing embryos develop a recognizable CAM. Discs, made by mixing test samples

with 1% (w/v) methylcellulose, are dried and placed on the CAM between major veins and approximately 0.5 cm from the embryo. Following another 48hour incubation at 37°C (1.5-3.5% CO_2), the samples are scored for their ability to inhibit angiogenesis. Inhibition appears as an avascular zone surrounding the implant and can often include elbows formed by veins avoiding the disc and a reduced number of capillaries in the region of the implant.

Although the invention has been described primarily in connection with special and preferred embodiments, it will be understood that it is capable of modification without departing from the scope of the invention. The following claims are intended to cover all variations, uses, or adaptations of the invention, following, in general, the principles thereof and including such departures from the present disclosure as come within known or customary practice in the field to which the invention pertains, or as are obvious to persons skilled in the field.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bouck, Noel P.
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Good, Deborah J.
Frazier, William A.

(ii) TITLE OF INVENTION: Method and Composition for Inhibiting Angiogenesis

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/841,656
(B) FILING DATE: 24-FEB-1992

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(A) APPLICATION NUMBER: US 07/464,369
(B) FILING DATE: 12-JAN-1990

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 92005-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (312)-456-8000
(B) TELEFAX: (312)-456-7776

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 239 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Arg Arg Pro Pro Leu Cys Tyr His Asn Gly Val Gln Tyr Arg Asn
1 5 10 15

Asn Glu Glu Trp Thr Val Asp Ser Cys Thr Glu Cys His Cys Gln Asn
20 25 30

Ser Val Thr Ile Cys Lys Lys Val Ser Cys Pro Ile Met Pro Cys Ser
35 40 45

Asn Ala Thr Val Pro Asp Gly Glu Cys Cys Pro Arg Cys Trp Pro Ser
50 55 60

Asp Ser Ala Asp Asp Gly Trp Ser Pro Trp Ser Glu Trp Thr Ser Cys
65 70 75 80

Ser Thr Ser Cys Gly Asn Gly Ile Gln Gln Arg Gly Arg Ser Cys Asp
85 90 95

Ser Leu Asn Asn Arg Cys Glu Gly Ser Ser Val Gln Thr Arg Thr Cys
100 105 110

His Ile Gln Glu Cys Asp Lys Arg Phe Lys Gln Asp Gly Gly Trp Ser

115 120 125

His Trp Ser Pro Trp Ser Ser Cys Ser Val Thr Cys Gly Asp Gly Val
130 135 140

Ile Thr Arg Ile Arg Leu Cys Asn Ser Pro Ser Pro Gln Met Asn Gly
145 150 155 160

Leu Pro Cys Glu Gly Glu Ala Arg Glu Thr Lys Ala Cys Lys Lys Asp
165 170 175

Ala Cys Pro Ile Asn Gly Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile
180 185 190

Cys Ser Val Thr Cys Gly Gly Gly Val Gln Lys Arg Ser Arg Leu Cys
195 200 205

Asn Asn Pro Ala Pro Gln Phe Gly Gly Leu Asp Cys Val Gly Asp Val
210 215 220

Thr Glu Asn Gln Ile Cys Asn Lys Gln Asp Cys Pro Ile Asp Gly
225 230 235

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Arg Arg Pro Pro Leu Cys Tyr His Asn Gly Val Gln Tyr Arg Asn
1 5 10 15

Asn Glu Glu Trp Thr Val Asp Ser Cys
20 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Ile Pro Pro Ile Thr Cys Val Gln Asn Gly Leu Arg Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Pro Trp Ser Ser Ala Ser Val Thr Ala Gly Asp Gly Val Ile Thr
1 5 10 15

Arg Ile Arg

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Pro Trp Asp Ile Ala Ser Val Thr Ala Gly Gly Gly Val Gln Lys
1 5 10 15

Arg Ser Lys

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Thr Xaa Xaa Xaa Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Pro Trp Xaa Xaa Xaa Ser Val Thr Xaa Gly Xaa Gly Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Thr Xaa Xaa Xaa Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Pro Trp Xaa Xaa Xaa Ser Val Thr Xaa Gly Xaa Gly Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Thr Xaa Xaa Xaa Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Pro Trp Xaa Xaa Xaa Ser Val Thr Xaa Gly Xaa Gly Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Xaa Gly Val Gln Tyr Arg Xaa
1 5

CLAIMS

We claim:

1. A method of inhibiting angiogenesis in a human patient, comprising administering to the patient a vascularization inhibitor comprising a peptide capable of inhibiting vascularization.

2. The method of claim 1 in which the patient is being treated for an internal tumor, and said inhibitor before administration is admixed with a slow release agent and thereafter a portion of the mixture is implanted in or adjacent to the tumor.

3. The method of claim 2 in which the tumor being treated is a skin cancer and said inhibitor before administration is admixed with a topical vehicle and thereafter applied to the surface of the skin cancer.

4. In the treatment of human patients having growing solid tumors with associated neovascularization, the method of retarding tumors growth comprising administering to the site of the patient's tumor a vascularization inhibitor comprising a peptide

capable of inhibiting vascularization, said inhibitor being applied to the tumor in an amount effective for retarding its enlargement.

5. A therapeutic product for controlling angiogenesis, comprising implantable pellets composed essentially of a slow release agent in admixture with a vascularization inhibitor comprising a peptide thereof capable of inhibiting vascularization.

6. A therapeutic product for controlling angiogenesis, comprising a topical vehicle in admixture with a vascularization inhibitor comprising a peptide capable of inhibiting vascularization.

7. A therapeutic product for controlling angiogenesis, comprising a parenteral therapeutic vehicle containing a vascularization comprising a peptide capable of inhibiting vascularization.

8. The therapeutic preparations of claims 5, 6 and 7 in which said inhibitor contains a region capable of inhibiting angiogenesis as determined by the rat corneal assay.

9. A therapeutic preparation for controlling angiogenesis, comprising a therapeutic vehicle containing a vascularization inhibitor comprising a peptide capable of inhibiting vascularization.

10. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from Sequence Id. No. 1.

11. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from Sequence Id. No. 2.

12. The composition of claim 11 wherein said peptide ranges from amino acid numbers 1-15.

13. The composition of claim 11 wherein said peptide ranges from amino acids numbers 11-25.

14. A composition capable of inhibiting vascularization which consists essentially of peptides having the amino acid sequences:

X - Gly - Val - Gln - Tyr - Arg - X

(Sequence Id. No. 12) wherein X are amino acids which do not destroy or interfere with inhibition of vascularization.

15. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from sequence Id. No. 3.

16. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from sequence Id. No. 4.

17. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from sequence Id. No. 5.

18. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from Sequence Id. No. 6 wherein X are amino acids which do not destroy or interfere with inhibition of vascularization.

19. A composition capable of inhibiting vascularization consisting essentially of amino acids selected from Sequence Id. No. 7 wherein X are amino acids which do not destroy or interfere with inhibition of vascularization.

20. A composition capable of inhibiting vascularization consisting essentially of a peptide mimic off TSP-I gene.

21. A composition capable of inhibiting vascularization consisting essentially of a peptide mimic off TSP-II gene.

22. The composition of Claims 10-19 wherein said composition is linked to a chemical carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01652

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 7/06, 7/08, 7/10, 13/00
US CL :514/8, 12, 13, 14, 16
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/8, 12, 13, 14, 16, 21; 530/324, 326, 327, 329, 380, 381, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

A-Geneseq 9, PIR 34, Swiss-Prot 23
search terms: SEQ ID NOS 2, 3, 6, 7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,585,754 (Meisner et al.) 29 April 1986, see the Abstract.	22
X Y	US, A, 4,898,732 (Fernandez) 06 February 1990, column 1, lines 45-59 and 64-68, column 2, lines 9-15 and 29-56.	20,21 1-8,22
X,E Y	US, A, 5,190,918 (Deutch et al.) 02 March 1993, column 6, lines 45-60, column 11, lines 8-14.	1,4,9,18,19 2,3,5-8,22
X,E	US, A, 5,192,744 (Bouck et al.) 09 March 1993, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 April 1993	Date of mailing of the international search report 14 APR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01652

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	N.P. Bouck et al., "Current Communications In Molecular Biology", published 1989 by Cold Spring Harbor Laboratory Press (NY), "Suppressor Control of an Inhibitor of Angiogenesis", pages 179-183, see entire document.	9-21 1-8,22
X Y	The Journal Of Cell Biology, Volume 112, No. 5, issued March 1991, C.A. Prater et al., "The Properdin-like Type I Repeats of Human Thrombospondin Contain a Cell Attachment Site", pages 1031-1040, see page 1037, Figure 8.	19 22
X	The Journal Of Biological Chemistry, Volume 265, No. 11, issued 15 April 1990, M.K. Wirtz et al., "In Vivo and in vitro Noncovalent Association of Excised α1(I) Amino-terminal Propeptides with Mutant pNα2(I) Collagen Chains in Native Mutant Collagen in a Case of Ehlers-Danlos Syndrome, Type VII", pages 6312-6317, see Figures 4 and 6.	15, 22